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Colloids engineering and filtration to enhance the sensitivity of paper-based biosensors

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ABSTRACT

Paper-based biosensors represent a disruptive technology by providing instantaneous and low-cost diagnostics for health and environmental applications. The lack of sensitivity can be an obstacle for this technology to compete with traditional analytical instrumentations. Aiming to improve the sensitivity of a paper-based colorimetric biosensor, we have applied colloids engineering in combination with filtration to lower the paper substrate backgrounds and optimize the immobilization of bio-molecules on paper. A model system consisting of an enzyme, alkaline phosphatase (ALP), and an inorganic colloid, calcium carbonate (CC), flocculated by a cationic dimethylamino-ethyl-methacrylate polyacrylamide (CPAM), demonstrated that the optimized CC flocs are best for enhancing the detecting sensitivity of ALP. The CC floc structure on paper was optimized by modulating its structure in suspension. Subsequently, the filtration process and the wicking ability of paper enabled to freeze the deposited CC structure inherited from the suspension. The incorporation of biomolecules into the CC before immobilizing on paper through filtration provided not only a better microenvironment, but also a higher surface density of immobilized biomolecules. The ALP detection limit of 117 fmol per zone (5 mm circle) in the current study was fifty times lower than that of the common soaking method for biomolecule immobilization. The minimum amount of biomolecules per unit substrate area required for detection was lowered by over an order of magnitude, compared with spotting methods (i.e. inkjet printing). The improvement was also demonstrated by the steepest slope of standard curve, the lowest background, and the highest activity of the bioactive paper probed with the diluted BCIP/NBT liquid substrates.

1. Introduction

Paper has recently attracted a considerable interest as means for developing low cost analytical devices as it is affordable, abundant, disposable, of high surface to volume or weight ratio, able to wick fluids, and easy to functionalize [1–7]. By providing instantaneous and low-cost diagnostics, paper-based biosensors represent a disruptive technology for health and environmental applications [8]. For paper-based biosensor using colorimetric assays, the prototype has evolved from a simple dipstick and 1D capillary-driven lateral flow system to more complicated 2D or 3D microfluidic paper-based analytical devices (μPADs) and microzone plates including paper-based enzyme-linked immunosorbent assay (P-ELISA). The biosensors enable multiplex and quantitative analysis, in addition to the conventional qualitative "yes/no" answers [5]. However, the low sensitivity and high limit of detection are preventing the paper-based analytical devices from reaching their full potential and compete with the traditional analytical instrumentation [5,9,10].

Immobilization of the biorecognition element (i.e. enzyme and antigen) on paper is the first step common to all colorimetric paper-based biosensors. The colour, often based on the traditional assays for spot-test analysis [11] and bio-recognition [12,13], provides a strong contrast with the whiteness of the paper. Consequently, the change in colour signal at the biorecognition zone is used to quantify the target analyte. Immobilization strategies of the biorecognition element (biomolecule) plays a significant role in determining sensitivity as it affects the conformation and distribution of biomolecules immobilized on paper [14,15]. Therefore, a better control of biomolecule immobilization on paper substrate has the potential for increasing sensitivity. In addition, the immobilization process that requires the smallest amount of biomolecules for the desired detecting sensitivity provides a significant economical advantage as the biomolecules can be scarce and are often the most expensive component of the biosensor [16].

Spotting through a pipette or an inkjet printer and soaking by either dipping or incubating paper in biomolecule solution are the common processes for biomolecule immobilization on paper [1,14,16,17]. While convenient, these methods suffer from a lim-

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ited control of the reagent solution flux through paper, and of the ratio: volume of biomolecule reagent solution to paper area. Most often, the flux of reagent solution and the immobilized biomolecule distribution largely depend on the liquid wicking paper, the physical–chemical properties of biomolecules, and the rheology and volume of the reagent solution. None of these existing methods can robotically and rapidly remove the unabsorbed reagent solution. In addition, spotting methods usually demand a high biomolecule concentration, 0.1–1 mg/mL, or at least 1.9 pmol of ALP per detecting zone (a circular area of a diameter of 5 mm) [18], although the biomolecule retention could reach 100% and the reagent volume required for quantitative biosensor can be very low (i.e. 3 μL). Whitesides' group has recently improved the detection sensitivity of rabbit IgG by using an indirect P-ELISA [9]. The detection limit per detecting zone was lowered to 54 fmol (or 81 ng); however, this is approximately 10 times higher than that obtained from a traditional ELISA using 96-well plates [9]. Soaking normally requires both large volumes of reagent and high concentrations of biomolecules [17]. As a result, large amounts of biomolecules are required for quantitative biosensors.

Developments in materials science and chemical engineering are offering new opportunities for enhancing sensor sensitivity [5]. For instance, the colour changes of gold nanoparticles, dependent on their degree of aggregation, have been successfully applied to colorimetric paper-based biosensors [19]. In fact, colloidal particles have been recognized as versatile and efficient templates for immobilization of biomolecules since the early 1980s. Particles, such as latex, microgels and silica, have already been used to fabricate bioactive papers for biosensing [16]. However, the inorganic particles flocculated at different degrees by polyelectrolyte have not been studied for the immobilization of functional biomolecules for paper-based biosensors. The porous structure of the flocs consisting of solid particles [20] can be manipulated by controlling the flocculation conditions. The flocs with controlled pore structures can serve as an alternative to encapsulate biomolecules, in a similar way to polymeric microcapsules. As a result, a wide range of biocompatible biominerals [21], such as calcium carbonate and calcium phosphates, can be incorporated into bioactive papers. Compared with a three-dimensional sol–gel network for biomolecule encapsulation [3], the suspension containing flocs of inorganic particles has a lower viscosity and allows to concentrate biomolecules through quick separation from the continuous liquid phase (i.e. water). By comparison, a biochemical immobilization [22] often requires hours of incubation and have diffusion limitation issues for the substrate (i.e. cells) to reach the immobilized biosorbent (i.e. enzyme).

The current study aims at engineering controlled colloid flocs incorporating biomolecules (colloids engineering) to increase the sensitivity of paper-based biosensor (bioactive paper). Two hypotheses relying on controlled colloid flocs were tested. The first was that the retention efficiency of biomolecules increases by retaining particle-biomolecule flocs through filtration; the second was that the activity of an immobilized biomolecule improves when incorporated within the biocompatible flocs.

An enzyme, alkaline phosphatase (ALP), was selected as model biomolecule as it produces a colour change from light yellow to dark blue, when in contact with the liquid substrate, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) [23]. Such a drastic colour change enables to visualize and to quantify the bioactivity of the target biomolecule (ALP) [17]. Calcium carbonate (CC) was chosen as a model colloid as it is widely used as filler in many industrial applications, including medicine and biotechnology. There are also interests in the application of CC for microcapsule fabrication, pharmaceutics [24–26] and stem cell tissue regeneration [27]. With great biocompatibility, calcium carbonate intensifies enzyme performance [28]. For ALP, the calcium divalent ions released from the calcium carbonate could enhance ALP activity [29]. The alkaline environment provided by the CC ($pK_a = 9$) is also hypothesized to enhance its bioactivity by retaining the pH at its optimum values (pH 7.5–9.5) [30]. In addition, calcium carbonate is widely used as pigment in the paper industry and contributes to the paper whiteness [31,32]. Such improved whiteness can lower the optical background signal and thus enhances the contrast between the paper substrate and the blue colour formed by ALP-BCIP/NBT interaction. The controlled CC flocs incorporating biomolecules will be obtained through controlling the adsorption kinetics of a cationic polyacrylamide on CC particles and be retained by a filtration process.

2. Experiments

2.1. Chemicals and materials

All chemicals and materials were from commercial resources and used as received. Alkaline phosphatase (ALP, from bovine intestinal mucosa, lyophilized powder, ≥10 DEA units/mg solid), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate, p-nitrophenyl phosphate (pNPP) liquid substrate, tris (hydroxymethyl) aminomethane, magnesium chloride, diethanolamine, hydrochloric acid and sodium hydroxide were all purchased from Sigma–Aldrich. Calcium carbonate (CC, precipitated, Schaefer Precarb 100 with a specific surface area (BET) of $8 \pm 2\ m^2/g$ and average particle, size d_{50} (Sedigraph), at $1.0 \pm 0.2\ \mu m$) was generously provided by Schaefer Kalk (Malaysia) SDN.BHD. Cationic dimethylamino-ethyl-methacrylate polyacrylamide (CPAM) (F1, SnowFlake Cationics, 13 MDa, 40 wt% charge density) was generously supplied by AQUA + TECH, Switzerland. Whatman® filter paper (Grade 5) was used as paper substrates for all studies.

2.2. Solution and suspension preparation

2.2.1. Buffer

The pH 9.8 buffer containing 1 M diethanolamine and 0.50 mM $MgCl_2$, adjusted by 5 M HCl, was used to prepare the ALP stock solution as per the Sigma–Aldrich's instruction. Two to four times concentrated buffer was also made for the convenience of dilution. The pH 9.2 buffer containing 10 mM Tris–HCl and 59.3 mM $MgCl_2$, as per the Sigma–Aldrich's instruction, was used to dilute the BCIP/NBT liquid substrate.

2.2.2. Liquid substrate

A series of diluted BCIP/NBT liquid substrates were prepared by mixing the liquid substrate from the supplier with the pH 9.2 tris buffer into 1/20, 1/15, 1/10 and 1/5 dilutions. The pNPP liquid substrate solution was prepared by dissolving the tablet set in the required volume of MilliQ water as per the manufacturer instructions.

2.2.3. Calcium carbonate suspension and flocculation

A stock solution of CPAM (1 mg/mL) was prepared on the day of experiment. 200 ppm CC suspensions under constant stirring at 100 rpm were ready for mixing with the ALP solution for its immobilization on paper substrates, after 10 min of ultrasonication. To obtain CC flocs, the pre-determined amount of CPAM (2 or 15 mg of CPAM/g of CC) was added quickly into the CC suspension under stir and the lapse time was monitored. The CC flocs sampled at the pre-determined lapse time were used for mixing with the ALP solution for its immobilization on paper substrates.

2.2.4. ALP solution or suspension

The pure ALP buffer solution (S), buffered suspension with CC colloids (CC) or with CC flocs induced by CPAM (CC flocs) was mixed with the ALP solution to obtain the required ALP concentration and the ratio of ALP buffer to CC mass. Typically, around 2 mg of CC was used in the 15 mL of suspension containing various amounts of ALP.

2.3. Quantification of ALP in solution

A pNPP liquid substrate was used during quantification of the ALP in solution, where its application protocol recommended for ELISA by the supplier was modified. The optimum condition was the addition of 2.5 mL of pNPP solution into the ALP buffer, resulting in the total volume of 3.6 mL. The mixture was then left to react in the dark for 30 min, immediately followed by the addition of 0.4 mL of 6 N NaOH to stop the reaction. The absorbance of the resultant yellow solution, measured by the UNICAM 8625 UV/visible spectrometer at 405 nm, was used to quantify the ALP in solution. The coefficient of variation (CV) of ALP concentration was less than 4.9% ($n = 4$).

2.4. Characterization of CC flocculation induced by CPAM

Characterization of CC flocculation was conducted as previously reported [33]. Briefly, after 10 min of ultrasonication, 200 ppm CC suspension stirred at 100 rpm was pumped through the photocell of the Photometric Dispersion Analyzer (PDA 2000, Rank Brothers) at a constant flow rate (30 mL/min). The early CC flocculation was monitored continuously by recording the ratio (R) of the root mean square of the fluctuating light intensity to the average transmitted light intensity. When the CC floc size goes beyond the PDA detecting limit (150–200 μm) at the late flocculation stage, the Focused Beam Reflectance Measurement (FBRM) was used to characterize the CC flocs.

2.5. Characterization of CPAM adsorption kinetics on CC

The CPAM adsorption kinetics was measured by quantification of the adsorbed polymer of the CC suspension sampled at different flocculation times through subtracting the amount of CPAM in the supernatant after centrifugation from that of the dosed polymer. The quantification of CPAM was made through titration (MütekTM PCD-03, BTG Instruments GmbH) after complete hydrolyzation, using 0.0005 N poly-diallyldimethylammonium chloride. The detection limit was below 0.1 μg (CPAM)/mL [33] and the coefficient of variation (CV) of the concentration of CPAM was less than 7.8% ($n = 3$).

2.6. Immobilisation of ALP on paper

The filter paper ($\phi = 55$ mm) was placed in the Buchner funnel with a sintered disc (G3). The 15 mL of buffer solution (S) or CC suspension (CC or CC flocs) containing ALP was then poured on top of the paper and left to soak for 10 s. The vacuum was turned on afterwards for 10 s to remove the excess liquid. The filtrate was collected for the ALP quantification when required.

2.7. Analysis of CC structure on paper

The paper with or without deposited calcium carbonate was dried at least overnight. The samples were then coated with carbon prior to analysis by the Scanning Electron Microscope (SEM, JEOL JSM-840A).

2.8. Quantification of immobilized ALP on paper

The amount of immobilized ALP on paper was determined by subtracting the quantity of ALP in the filtrate from the added amount of ALP, using a pNPP liquid substrate.

2.9. Evaluation of activity of bioactive paper

Three microlitres of the BCIP/NBT liquid substrate (or diluted liquid substrates) was spotted on the paper substrate immobilized with ALP, which was then left in the dark for 30 min for the blue colour formation. The quantitative colorimetric detection of the immobilized ALP was made by detecting the reflectance of colour zone (EPSON PERFECTION 2450 scanner and Image J software) at the resolution of 600 dpi [17]. The RGB pixels was converted into the brightness values (V) using the formula ($V = 0.299R + 0.587G + 0.114B$) [34]. The lower V value represents more blue colour development, or a darker colour in gray scale. The activity of the bioactive paper (D_V) was evaluated by the brightness value difference obtained by subtracting the V value of the paper immobilized with ALP from the V value of the reference paper substrate (background). The D_V value varies with the concentration of the added ALP on paper substrate, the ALP immobilization condition and the dilution factor of enzymatic liquid substrate. Therefore, comparison of the D_V value is subject to the defined constraints of circumstances. The average D_V from multiple measurements ($n = 7$) was used and the confidence interval was calculated using the standard derivation at the probability level of 95%.

3. Results and discussion

3.1. Quantification of ALP in solution

An estimation of biomolecule immobilization efficiency during the preparation of biosensors is essential for the economic optimization of low cost analytical devices. This evaluation was made by measuring the difference in concentration of the ALP in solution before and after filtering through paper. Therefore, the quantification of ALP in solution was investigated.

Using the BCIP/NBT substrate to directly quantify the soluble ALP is problematic as the analysis is based on the blue colour generated from an insoluble NBT diformazan [23]. The insoluble precipitate scatters light and thus interferes with the quantification of the soluble ALP by spectrophotometry. However, the reaction of ALP with a pNPP substrate forms a soluble yellow p-nitrophenol, which is recommended to quantify the amount of ALP in ELISA by the supplier. To adapt the protocol for analysis of the ALP in a test tube, the optimum ratio of the pNPP to ALP was investigated. An adsorption plateau at 405 nm was found at the ratios of pNPP (mL) to ALP (mg), ranging from 2000 to 10,000. A 2.5 mL pNPP in the total of 4 mL buffer with the addition of 0.6 N NaOH at the end of the reaction was thus chosen to quantify the ALP concentration in solution. The standard curve demonstrated a good linear relationship between the absorbance (Abs) and the ALP concentration (Fig. 1) ($R = 0.9990$).

3.2. Incorporation of calcium carbonate to optimize bioactive paper activity

The hypothesis that calcium carbonate enhances ALP bioactivity is mainly based on its chemical composition [29], alkaline microenvironment [30], increased adsorption of ALP and pore structure of its flocculated aggregates. The electrostatic interaction between CC and ALP often described in colloid chemistry played little role in the hypothesis. This is because the CC, having a low negative

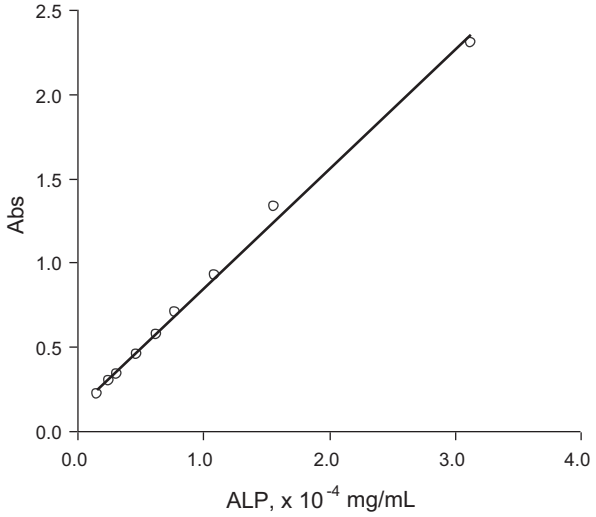


Fig. 1. The standard curve for quantification of ALP in solution through its reaction with a pNPP substrate (405 nm).

zeta potential (-1.65 ± 0.17 mV at pH 9.7 ± 0.3) [33], facilitates the adsorption of ALP on paper, regardless of the negative charge of the ALP (isoelectric point: 4.4–5.8) [35] under alkaline conditions. A filtration process was investigated to control the microenvironment of an immobilized ALP on paper substrates by incorporating calcium carbonate either as colloids or as flocs of controlled structure. The ratio of the buffer volume (pH 9.8) to the amount of CC (mg) present in the 15 mL suspension aliquot affected the activity of the bioactive paper at a fixed ALP loading concentration (0.0125 mg/mL) (Fig. 2). Such an effect may result from the interaction of the CC with the ALP enzyme instead of a change in the pH, as the addition of CC at the ratio ranging from 3.5 to 22.0 did not significantly change the pH of the 15 mL suspension aliquot. In addition, the activity of bioactive paper made with CC suspensions free of buffer was rather low. Therefore, the hypothesis that calcium carbonate improves the ALP bioactivity by providing an alkaline microenvironment is rejected. The ALP buffer (pH 9.8)/CC (mg) ratio of 5.3 was selected for the incorporation of CC colloids and CC flocs in the subsequent ALP immobilization studies.

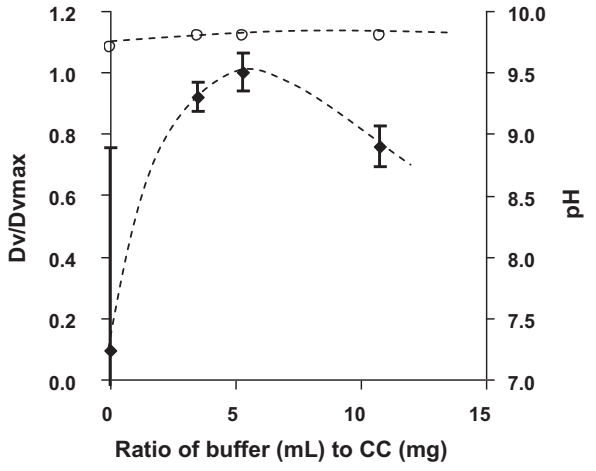


Fig. 2. Effect of the ratio of buffer (pH 9.8) volume to the mass of calcium carbonate on the system pH (○) and the activity of bioactive paper (●) (ALP in the detection cell: 7.8×10^{-5} mg/mL). $D_{v\,max}$ represents the maximum bioactivity observed in this study.

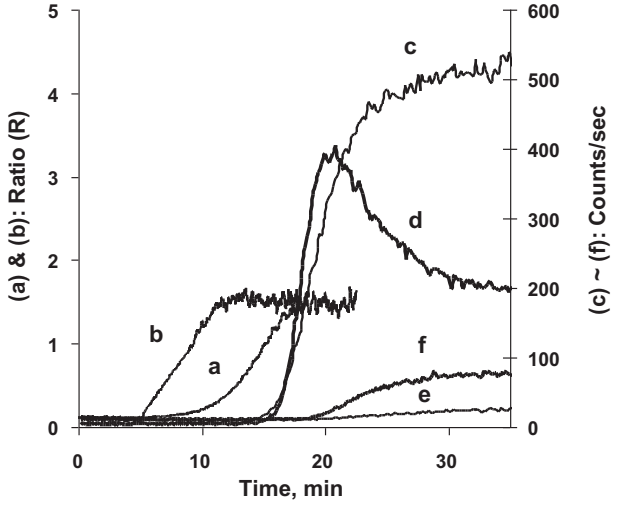


Fig. 3. PDA ratio signal (R) of the CC suspensions as a function of time at different CPAM dosages of (a) 2 and (b) 15 mg of CPAM/g of CC, respectively, and the number of counts evolution in FBRM for the CC suspensions at a size range of either (c and d) 50–150 μm or (e and f) 150–300 μm at the CPAM dosages of (c and e) 2 and (d and f) 15 mg of CPAM/g of CC, respectively. CPAM was injected at 5 min.

3.3. Effect of CC flocculation on bioactive paper activity

Two complementary techniques, Photometric Dispersion Analyzer (PDA) and Focused Beam Reflectance Measurement (FBRM), were used to quantify the full flocculation process of CC induced by the CPAM, from colloids to micro-flocs. The CC early flocculation was monitored by PDA as a function of time at two different polymer concentrations (2 and 15 mg of CPAM/g of CC) (Fig. 3). The growth rate of CC flocculation at a low polymer dose gradually increased after an induction period and remained steady (13–17 min) before approaching a flocculation plateau. In contrast, a high polymer dose (15 mg of CPAM/g of CC) instantly induced CC flocculation, which proceeded at an almost constant flocculation rate and reached the plateau at around 12 min. The absolute PDA ratio (R), an indication of the CC flocs structure, varied with the polymer dose and the flocculation time. In the late flocculation stage, the FBRM counts, indicative of particle numbers, as a function of time for the particle size ranging between 50 and 150 μm and between 150 and 300 μm, respectively, disclosed the continued progressive flocculation of CC suspension. However, the floc sizes were different for the low and high CPAM doses (Fig. 3). Therefore, both the CPAM dose and the flocculation time of the CC flocs were controlled to modulate the CC flocs structure for ALP immobilization.

The concentration of a cationic polymer was previously found to affect the antibody performance during its application for pathogen detection, where a low polymer concentration was beneficial [36,37]. Therefore, the amount of free polymer left in suspension and the amount of adsorbed polymer on CC were quantified (Fig. 4). For a low dose of polymer (2 mg of CPAM/g of CC), the amount of free CPAM remained almost constant up to 6 min after polymer addition; afterwards, the free CPAM depletion kinetics demonstrated similar trends to that at a high CPAM dose: an initial linear decrease up to around 10 min followed by a plateau (0.6 mg of CPAM/g of CC). The corresponding CPAM adsorption plateaus are 1.7 and 13.6 mg of CPAM/g of CC for the initial CPAM dosages of 2 and 15 of CPAM/g of CC, respectively. We observed discrepancies between the flocculation rates and the predictions from the surface coverage theory based on a simple monolayer adsorption model (around 3.9 mg CPAM/g CC) [38,39] at both the early and late flocculation stages.

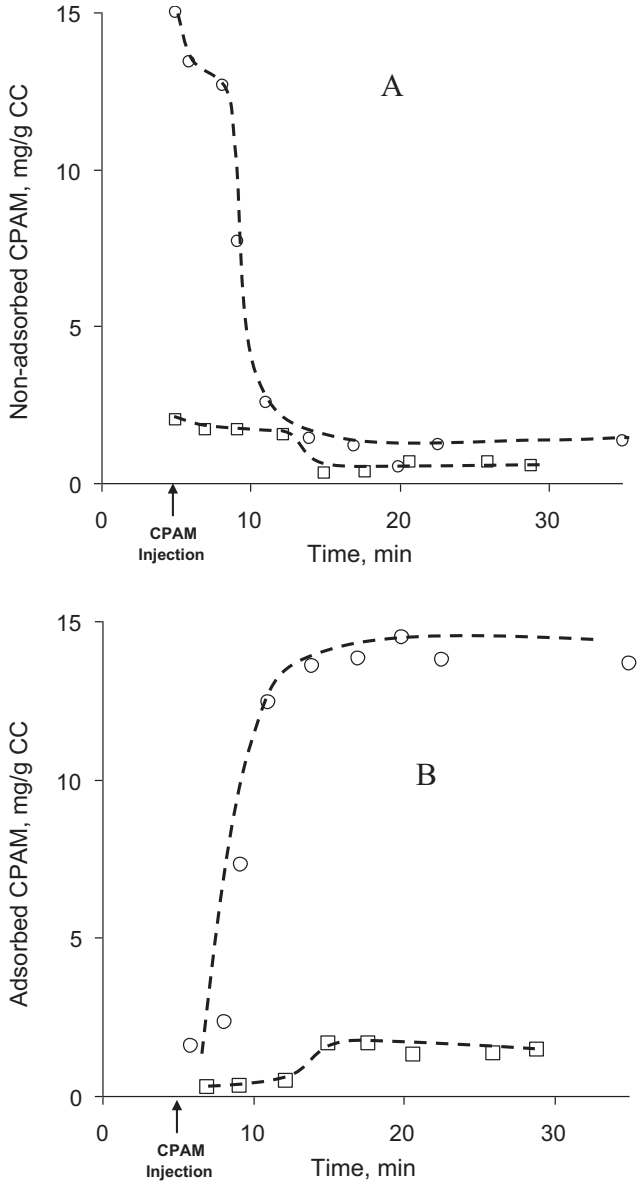


Fig. 4. (A) Non-adsorbed (free) CPAM depletion kinetics and (B) CPAM adsorption kinetics during CC flocculation at the CPAM doses of (□) 2 mg and (○) 15 mg of CPAM/g of CC, respectively. CPAM was injected at 5 min.

Comparison of Figs. 3 and 4 disclosed that all early stages of CC flocculation detectable by PDA happened under non-equilibrium polymer adsorption regimes. The full analysis is available elsewhere [33]. Such a coincidence indicates that the enzyme immobilization on substrates through colloids and flocs requires the development of procedures which facilitate the retention of their dynamic structures formed during the kinetic process. The filtration process effectively fixed, on paper, the CC structure inherited from the engineered flocs of the CC suspension (Fig. 5). The CC flocs showed larger aggregates on paper than those from the CC colloids. This CC structure affected the activity of the bioactive paper (Fig. 6). Statistical *t*-tests based on the two samples suggested that the ALP incorporated with the CC flocs formed at either 9 min with 2 mg of CPAM/g of CC or 8 min with 15 mg of CPAM/g of CC presented a higher bioactivity as indicated by the larger D_v signal (1.3 times the non-flocculated CC colloids, D_0), assuming equal variances. Further investigation with a series of ALP (0.00625–0.075 mg of ALP/mL) incorporated with the CC flocs under the above-mentioned CC flocculation conditions disclosed that the low CPAM dose (2 mg of CPAM/g of CC) was superior; this is consistent with Wang's previous report [36]. The condition at 9 min with 2 mg of CPAM/g of CC demonstrated consistent larger D_v values of lower confidence intervals across the full ALP concentrations studied, which also showed a better linear relationship between the D_v signal and the added ALP concentration (data not shown). Therefore, the CC flocs formed at 9 min with 2 mg of CPAM/g of CC was selected to further study the ALP immobilization on paper.

3.4. Effect of immobilization conditions on ALP retention efficiency on paper

The incorporation of CC and CC flocs during the ALP immobilization process increased the enzyme immobilization efficiency across the full range of ALP concentrations investigated, regardless of the CC structures (Fig. 7). The average ALP loading efficiency by incorporation of CC either as a colloid or a floc increased to 27% from the previous 12% achieved only with ALP buffer. In addition, the loaded amount of ALP per unit paper surface increased linearly with the ALP concentration in the presence of CC colloids or CC flocs, whereas no significant increase of the loaded ALP was observed with the ALP concentration up to 0.0125 mg/mL in the absence of CC.

Although the increased surface area from incorporating CC particles could allow more ALP molecules directly adsorbed on paper, the easiness of the ALP molecule diffusion onto the CC particles in suspension also accounted for increasing the retention efficiency of ALP. Experiments disclosed that the ALP retention efficiency of the paper with the pre-deposited CC particles was the same as that of the bare paper, when the pure ALP buffer solution (0.002–0.05 mg/mL) was used for preparing the bioactive paper. This implied that the CC in suspension allowed easier diffusion of the enzyme onto the CC surface than the compact CC pre-deposited on paper. In addition, the similarity of the ALP immobilization efficiency between the CC colloids and the CC flocs suggests that the open porous structure of the CC flocs in suspension allowed full ALP molecules diffusion inside the pores (Fig. 5D).

3.5. Effect of ALP immobilization on bioactive paper activity

The incorporation of CC at different quantities and under various aggregation levels influences the roughness, brightness and light scattering of the paper substrate [40]. Nevertheless, the concept of the D_v value was defined to remove the effect of the background signal from the substrate and to represent only the activity of the bioactive paper. The standard curves for quantification of ALP (0–0.08 mg/mL) through forming bioactive paper were compared for various ALP immobilization conditions (Fig. 8), where the data for the pure ALP buffer (S) by soaking process was extrapolated [17] by using DataThief software [41]. At a fixed ALP loading concentration, a larger D_v value indicates an increased blue colour development, and thus a larger activity of the bioactive paper. Compared with the soaking method [17], the filtration process for ALP immobilization demonstrated significant improvements in the activity of the bioactive papers. Two reasons can be ascribed for such an improvement. The first is the absence of blotting procedure, a common practice for the soaking method, which prevented the loss of the superficial ALP molecules on paper substrates. The second is the force generated from the vacuum filtration process which could facilitate the ALP adsorption inside the capillaries of the paper.

All calibration curves across ALP concentration up to 0.08 mg/mL followed the expected nonlinear functions [1] and were best fitted with the 2nd order logarithm (Fig. 8A). Although the amount of immobilized ALP increased with enzyme concentration (up to 0.05 mg/mL) (Fig. 7), the blue colour almost saturated at the added ALP concentrations beyond 0.02 mg/mL, as indicated by the flat D_v

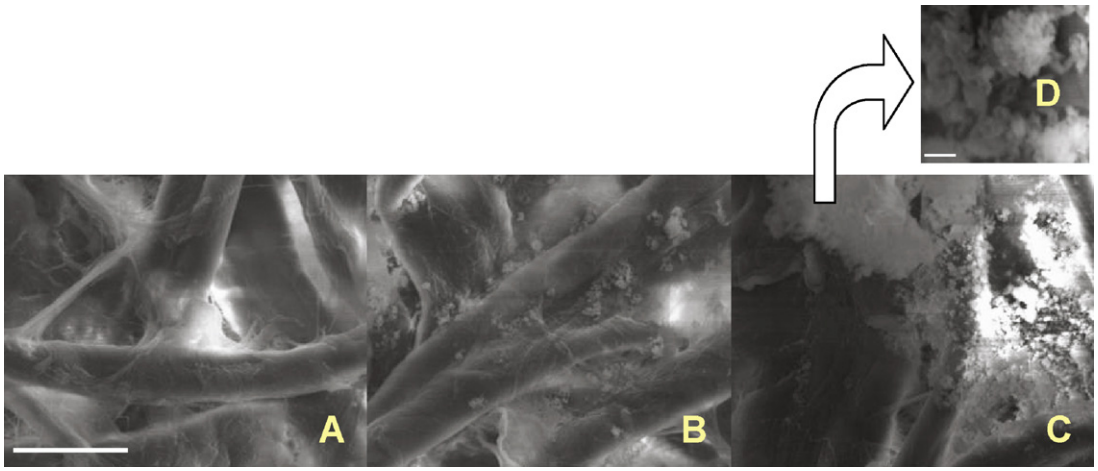


Fig. 5. Illustrated retention of the CC structure on paper substrate by SEM through the filtration process: (A) Surface of bare paper substrate, (B) CC suspension and (C) CC flocs. (D) is the magnified image of one spot of (C). The scale bar: (A)–(C): 100 μm, (D): 1 μm.

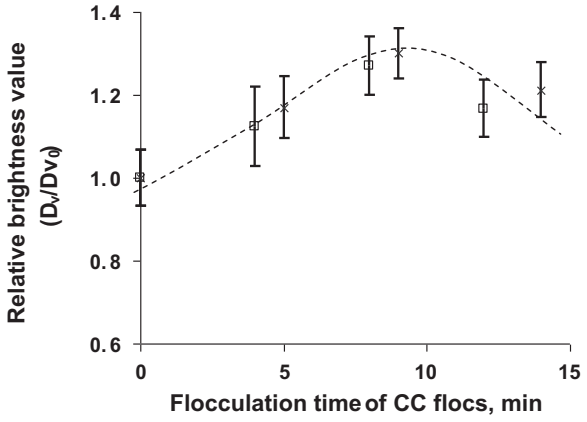


Fig. 6. The effect of flocculation time of CC flocs on the relative activity of the bioactive paper (relative to the D_v value obtained by use of CC colloids, D_{v0}) at the CPAM doses of (×) 2 mg and (□) 15 mg of CPAM/g of CC. The added ALP concentration in CC colloids or flocs was 0.0125 mg/mL and the CPAM was injected at 0 min.

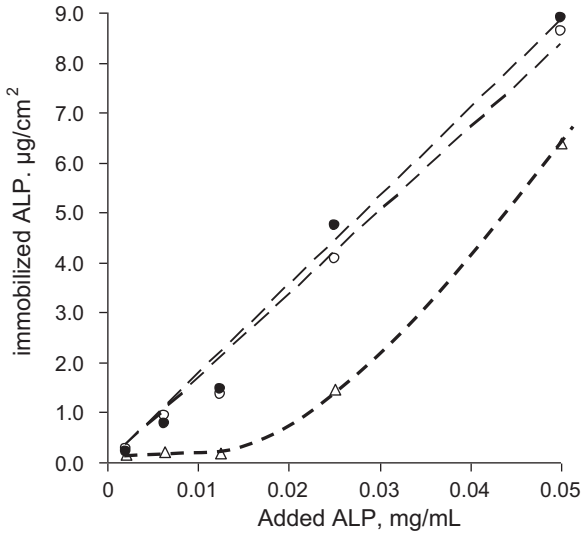


Fig. 7. Relationship between the amount of immobilized ALP and the concentration of added ALP under various immobilization conditions through filtration process: (△) buffer only (S), (○) CC colloids and (●) CC flocs.

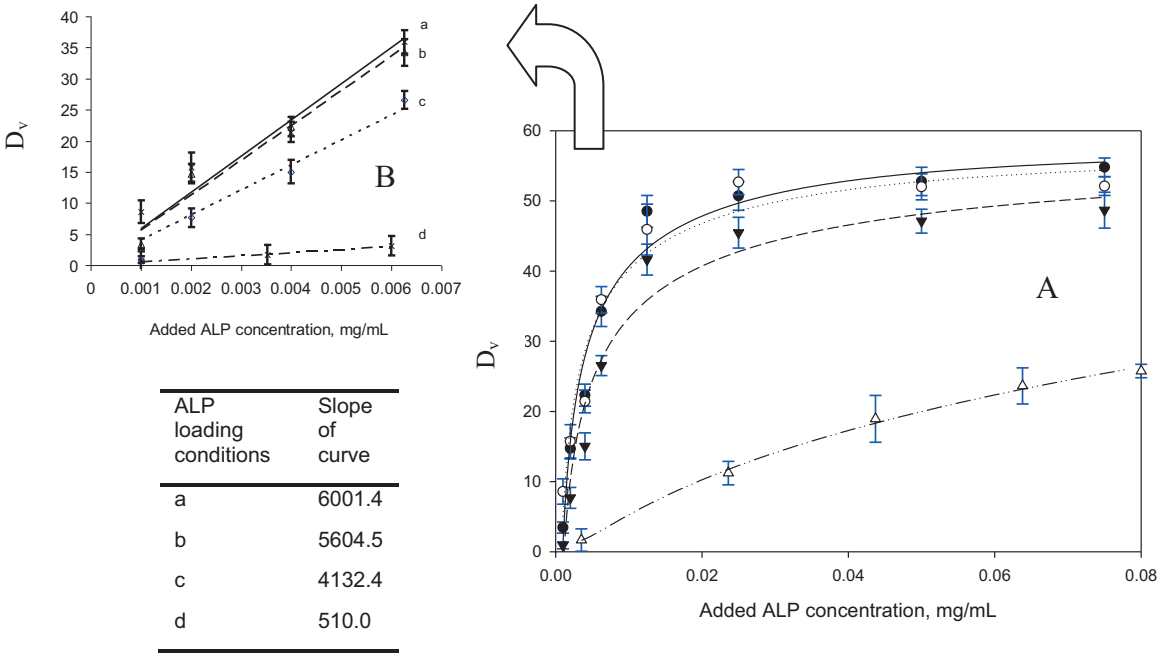
values. This could result from the limited access of the ALP that reacts with a drop of 3 μL of the BCIP/NBT liquid substrate at a certain depth of paper and the likelihood of blue colour detection by the scanner. The accessibility and conformation of an immobilized ALP and the surface morphology of bioactive papers were all affected by the ALP immobilization conditions. The smoother paper surface without calcium carbonate had the least amount of immobilized ALP and less colour reflection areas, and thus the lowest colour saturation values.

To further evaluate the sensitivity of the bioactive paper, the standard curves within the lower ALP concentrations were magnified (Fig. 8B). ANOVA analysis showed that all standard curves were linear at the ALP concentrations ranging between 0.001 and 0.00625 mg/mL. The highest slope in Fig. 8B implied that the CC flocs provided the best microenvironment to enhance the detecting sensitivity of ALP through forming bioactive paper.

3.6. Effect of ALP immobilization on its detection limit

To compare the detection limits of paper-based ALP sensors prepared under different conditions, the brightness values (V) and their variations of the respective background substrates were evaluated for different ALP immobilization conditions. The t-test for the two samples demonstrated that the V value from the background with the CC flocs ($V_{CC\ flocs} = 253.1 \pm 0.3$) was significantly larger than the V value from the background with the CC colloids ($V_{CC} = 252.4 \pm 0.3$), assuming equal variances at the 95% significance level. The same t-test also demonstrated that the V value from the background with the CC colloids was significantly larger than the V value from the background only using the buffer ($V_S = 251.2 \pm 0.3$). The larger V value represents a brighter background, thus, a better sensor supporting medium.

The detection limits of ALP were estimated by three times the estimated standard deviation of the respective blanks at the 95% significance level [42]. The CC flocs indicated a slightly lower limit (1.1 nM) than CC colloids (1.3 nM) and pure buffer (S) systems (1.9 nM). By considering the test zone of bioactive paper to be a 5 mm circle [9], the minimum amount of ALP required per zone were 117, 134, 194 and 6066 fmol for the CC flocs, CC colloids and S system by a filtration method, and S system by a soaking method (extrapolated from Khan et al. [17] and processed with DataThieft [41]), respectively. The incorporation of CC flocs combined with filtration method reduced the detection limit of ALP by a factor of 50 compared with the common soaking method (58 nM). The lowest detection limit in the current study is comparable with the best pre-



ALP loading conditions	Slope of curve
a	6001.4
b	5604.5
c	4132.4
d	510.0

Fig. 8. Standard curves for quantification of the ALP in solution through forming bioactive paper under different ALP immobilization conditions: (a) CC flocs (○), (b) CC colloids (●) and (c) S by filtration process (▼), as well as (d) S by soaking process (△), extrapolated form the reference using DataThief III [17,41]. (B) is the magnified figure of (A) at the lower ALP concentrations.

viously reported sensitivity of 54 fmol per zone for IgG [9]. Further improvement is also feasible by decreasing the ALP loading volumes. This demonstrated that combining colloids engineering with the filtration process offers great potential for improving sensor sensitivity.

3.7. Activity of bioactive paper probed by diluted BCIP/NBT substrates

An alternative method was also investigated to further evaluate the performance of bioactive papers. An insufficient amount of ALP liquid substrate consisting of a series of diluted BCIP/NBT was served as a probe to differentiate the activity of the bioactive papers made under various conditions at a fixed ALP concentration (0.0125 mg/mL). In agreement with the previous findings, the flocculation of CC affected the bioactivity of the immobilized ALP, despite its similar ALP immobilization efficiency to that of CC colloids. This confirms that the ALP conformation was affected by CC flocculation. The bioactive paper with the CC flocs on paper substrates again demonstrated the highest activity as indicated by the largest D_v value achieved at a given liquid substrate dilution factor (Fig. 9).

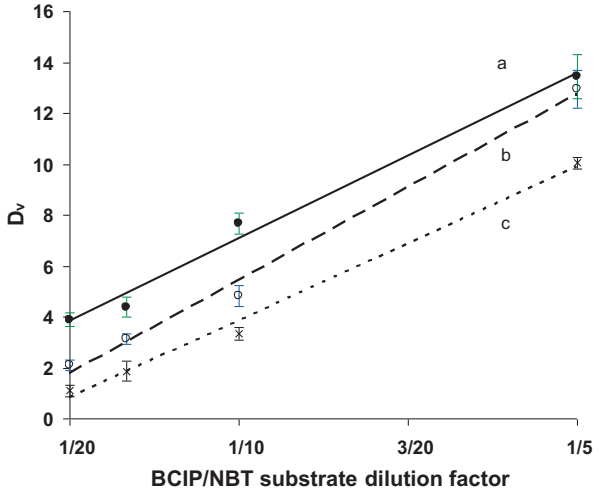


Fig. 9. Activity (D_v) of the bioactive paper probed by the BCIP/NBT substrates of different dilutions under various ALP immobilization conditions by filtration: (a) CC flocs, (b) CC colloids and (c) S. Added ALP concentration: 0.0125 mg/mL.

4. Conclusions

Colloids engineering combined with filtration improved the sensitivity of paper-based biosensors; this has been illustrated in this study by using a model system consisting of an enzyme (ALP) and an inorganic colloid: calcium carbonate (CC). The ALP molecules incorporated into the CC colloids or flocs were retained on paper through filtration. The optimized CC flocs were best for enhancing the sensor performance as demonstrated by the steepest slope of standard curve, the lowest background, the lowest detection limit (1.1 nM), and the highest bioactivity of an immobilized ALP. To better control the CC floc structure ideal for ALP immobilization, the full CC flocculation process induced by CPAM was analysed from colloids to large flocs by combining two complementary techniques: PDA and FBRM. Discrepancies between the measured CC flocculation rates and the prediction from the surface coverage theory based on the monolayer adsorption model were observed at both the early and the late flocculation stages. All early stages of flocculation detectable by PDA happened under non-equilibrium polymer adsorption regimes. In the progressive CC flocculation process, the polymer dose and the flocculation time were both used as variables to modulate the CC floc structure in suspension. The filtration process combined with the wicking ability of paper enabled to retain, on paper, the CC floc structure inherited from the suspension. The amount of immobilized ALP on paper was quantified by adapting the commercially available ALP-pNPP protocol routinely used for ELISA. The average ALP loading efficiency, by incorporation of CC either as colloids or as flocs through filtration, almost doubled to 27%. While such enzyme immobilization efficiency is lower than that achieved by printing (up to 100%), the ALP detection limit of

117 fmol per zone (5 mm circle) is over an order of magnitude lower than that achieved by printing (1.9 pmol per zone). Compared with the popular soaking method used for biomolecule immobilization in paper-based colorimetric biosensors, the filtration of preformed floc CC-ALP on paper investigated in this study achieved an ALP detection limit of 1.1 nM or 117 fmol per zone of 5 mm circle. This was fifty times lower, therefore, significantly more efficient, than soaking.

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References

[1] A.W. Martinez, S.T. Phillips, G.M. Whitesides, E. Carrilho, Diagnostics for the developing world: microfluidic paper-based analytical devices , Anal. Chem. 82 (2010) 3–10.
[2] E. Carrilho, S.T. Phillips, S.J. Vella, A.W. Martinez, G.M. Whitesides, Paper microzone plates , Anal. Chem. 81 (2009) 5990–5998.
[3] S.M.Z. Hossain, R.E. Luckham, M.J. McFadden, J.D. Brennan, Reagentless bidirectional lateral flow bioactive paper sensors for detection of pesticides in beverage and food samples , Anal. Chem. 81 (2009) 9055–9064.
[4] A.W. Martinez, S.T. Phillips, G.M. Whitesides, Three-dimensional microfluidic devices fabricated in layered paper and tape , Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 19606–19611.
[5] W.A. Zhao, A. van den Berg, Lab on paper , Lab Chip 8 (2008) 1988–1991.
[6] A.W. Martinez, S.T. Phillips, E. Carrilho, S.W. Thomas, H. Sindi, G.M. Whitesides, Simple telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-time, off-site diagnosis , Anal. Chem. 80 (2008) 3699–3707.
[7] R.W. Peeling, K.K. Holmes, D. Mabey, A. Ronald, Rapid tests for sexually transmitted infections (STIs): the way forward , Sex. Transm. Infect. 82 (2006) v1–v6.
[8] C.M. Christensen, M.E. Raynor, The Innovator's Solution: Creating and Sustaining Successful Growth , Harvard Business School Press, Boston, MA, 2003, p. 301.
[9] C.M. Cheng, A.W. Martinez, J. Gong, C.R. Mace, S.T. Phillips, E. Carrilho, K.A. Mirica, G.M. Whitesides, Paper-based ELISA , Angew. Chem. Int. Ed. 49 (2010) 4771–4774.
[10] E. Fu, P. Kauffman, B. Lutz, P. Yager, Chemical signal amplification in two-dimensional paper networks , Sens. Actuators B: Chem. 149 (2010) 325–328.
[11] E. Jungreis, Spot Test Analysis: Clinical, Environmental, Forensic, and Geochemical Applications , 2nd ed., Wiley, New York, 1997.
[12] E.D. Hilborn, W.W. Carmichael, M. Yuan, S.M.F.O. Azevedo, A simple colorimetric method to detect biological evidence of human exposure to microcystins , Toxicon 46 (2005) 218–221.
[13] N. van Poperin, D.E. Lopatin, Slot immunoblot assay for detection and quantitation of periodontal disease-associated microorganisms in dental plaque , J. Clin. Microbiol. 29 (1991) 2554–2558.
[14] O.P. Lehtonen, M.K. Viljanen, Antigen density in ELISA: effect on avidity dependency , J. Immunol. Methods 36 (1980) 63–70.
[15] H.A. Fields, C.L. Davis, D.W. Bradley, J.E. Maynard, Experimental conditions affecting the sensitivity of Enzyme-Linked Immunosorbent-Assay (ELISA) for detection of hepatitis-B surface-antigen (Hbsag) , Bull. World Health Organ. 61 (1983) 135–142.
[16] R. Pelton, Bioactive paper provides a low-cost platform for diagnostics , Trends Anal. Chem. 28 (2009) 925–942.
[17] M.S. Khan, X. Li, W. Shen, G. Garnier, Thermal stability of bioactive enzymatic papers , Colloid Surf. B: Biointerfaces 75 (2010) 239–246.
[18] S. Wang, C. Zhang, Y. Zhang, Lateral flow colloidal gold-based immunoassay for pesticide , in: A. Rasooly, K.E. Herold (Eds.), Biosensors and Biodetection: Methods and Protocols, vol. 2, Humana Press, New York, 2009, pp. 237–252.
[19] W.A. Zhao, M.M. Ali, S.D. Aguirre, M.A. Brook, Y.F. Li, Paper-based bioassays using gold nanoparticle colorimetric probes , Anal. Chem. 80 (2008) 8431–8437.
[20] M.G. Rasteiro, F.A.P. Garcia, M.d.M. Perez, Applying LDS to monitor flocculation in papermaking , Particul. Sci. Technol. 25 (2007) 303–308.
[21] S. Mann, Biomineralization: Principles and Concepts in Bioinorganic Materials Chemistry , Oxford University Press, New York, 2001.
[22] M. Tolba, O. Minikh, L.Y. Brovko, S. Evoy, M.W. Griffiths, Oriented immobilization of bacteriophages for biosensor applications , Appl. Environ. Microbiol. 76 (2010) 528–535.
[23] M.S. Blake, K.H. Johnston, G.J. Russelljones, E.C. Gotschlich, A rapid, sensitive method for detection of alkaline-phosphatase conjugated anti-antibody on western blots , Anal. Biochem. 136 (1984) 175–179.
[24] A.B. Joshi, R. Srivastava, Polyelectrolyte coated calcium carbonate microparticles as templates for enzyme encapsulation , Adv. Sci. Lett. 2 (2009) 329–336.
[25] D. Shan, M. Zhu, H. Xue, S. Cosnier, Development of amperometric biosensor for glucose based on a novel attractive enzyme immobilization matrix: calcium carbonate nanoparticles , Biosens. Bioelectron. 22 (2007) 1612–1617.
[26] D. Shan, M. Zhu, E. Han, H. Xue, S. Cosnier, Calcium carbonate nanoparticles: a host matrix for the construction of highly sensitive amperometric phenol biosensor , Biosens. Bioelectron. 23 (2007) 648–654.
[27] A. Champa Jayasuriya, A. Bhat, Mesenchymal stem cell function on hybrid organic/inorganic microparticles in vitro , J. Tissue Eng. Regen. Med. 4 (2010) 340–348.
[28] R. Rosu, Y. Iwasaki, N. Shimizu, N. Doisaki, T. Yamane, Intensification of lipase performance in a transesterification reaction by immobilization on $CaCO_3$ powder , J. Biotechnol. 66 (1998) 51–59.
[29] M. Besman, J.E. Coleman, Isozymes of bovine intestinal alkaline-phosphatase , J. Biol. Chem. 260 (1985) 1190–1193.
[30] M. Fosset, D. Chappele, M. Lazdunsk, Intestinal alkaline phosphatase physical properties and quaternary structure , Biochemistry 13 (1974) 1783–1788.
[31] A. Jääskeläinen, K.-E. Peiponen, U. Tapper, E.I. Kauppinen, K. Lumme, Effective refractive index of calcium carbonate pigment slurries by a surface-plasmon-resonance sensor , Dyes Pigments 52 (2002) 15–21.
[32] H. Holik, Handbook of Paper and Board , Wiley InterScience, 2006, pp. 38 and 58.
[33] P. Peng, G. Garnier, Effect of cationic polyacrylamide adsorption kinetics and ionic strength on precipitated calcium carbonate flocculation , Langmuir 26 (2010) 16949–16957.
[34] National Institutes of Health, Image-J, http://rsb.info.nih.gov/ij/docs/menus/analyze.html, April 10, 2010.
[35] Sigma–Aldrich, Alkaline phosphatase, http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase.html, 19 April 2011.
[36] J. Wang, R. Pelton, L.J. Veldhuis, C.R. MacKenzie, J.C. Hall, C.D.M. Filipe, Wet-strength resins and surface properties affect paper-based antibody assays , Appita J. 63 (2010) 32–36.
[37] M.S. Khan, S.B.M. Haniffa, A. Slater, G. Garnier, Effect of polymers on the retention and aging of enzyme on bioactive papers , Colloid Surf. B: Biointerfaces 79 (2010) 88–96.
[38] F. Mabire, R. Audebert, C. Quivoron, Synthesis and solution properties of water soluble copolymers based on acrylamide and quaternary ammonium acrylic comonomer , Polymer 25 (1984) 1317–1322.
[39] Y. Zhou, G.V. Franks, Flocculation mechanism induced by cationic polymers investigated by light scattering , Langmuir 22 (2006) 6775–6786.
[40] R. Farnood, Optical Properties of Paper: Theory and Practice , Pulp & Paper Fundamental Research Society, Bury, 2009, p. 273.
[41] Datathief, DataThief 3.0, http://download.cnet.com/DataThief/3000-2053_4-80439.html, August 15, 2010.
[42] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry , 2nd ed., Ellis Horwood PTR Prentice Hall, New York, 1993, p. 233.